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The Effect of Dietary Zinc on Vascular Function with Aging

Tsuzumi Kanaoka

University of Colorado Boulder

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The Effect of Dietary Zinc on Vascular Function with Aging

By
Tsuzumi Kanaoka

A thesis submitted in partial fulfillment of the requirements for graduation with

DEPARTMENTAL HONORS

From the department of
INTEGRATIVE PHYSIOLOGY

Examining Committee:

Dr. Douglas R. Seals, Thesis Advisor
Dept. of Integrative Physiology

Dr. David E Sherwood, Member
Dept. of Integrative Physiology

Dr. E. Christian Kopff, Member
Dept. of Honors Program

UNIVERSITY OF COLORADO AT BOULDER

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TABLE OF CONTENTS:

<i>Abstract</i>	3
<i>Acknowledgements</i>	4-5
I. Introduction	
Cardiovascular Disease and Aging	6
Dysfunction of Arteries.....	7
Dysfunction of Endothelial Cell.....	7-12
<i>Reduced Nitric Oxide Bioavailability</i>	
<i>Oxidative Stress</i>	
<i>“Inflamm-aging”</i>	
Dietary Zinc: Clinical Significance	13-14
Zinc Intake and Associated Conditions.....	14-15
Biological Mechanisms of Zinc.....	15-18
<i>Anti-Inflammatory and Antioxidant Roles</i>	
<i>Transcription Level Roles</i>	
Working Hypothesis.....	19
II. Materials and Methods	
Subjects.....	20
Study Procedures.....	21
Subject Characteristics and Group Selection.....	21-22
Vascular Health Measures Measurements.....	23-26
<i>Blood Assay</i>	
<i>Endothelium Dependent Dilation Measures</i>	
<i>Endothelium Independent Dilation Measure</i>	
<i>Pulse Wave Velocity</i>	
<i>Statistical Analysis</i>	
III. Results	
Subject Characteristics.....	27-28
Blood Assay.....	28-33
EDD and EID.....	34
Pulse Wave Velocity.....	34
IV. Discussion	
Biomedical Importance.....	35-36
Inconsistency and Variability.....	36-37
Limitations of the Study.....	38
Strength of the Study.....	39
V. Conclusion	39
Perspectives.....	39-40
VI. References	41-42
VII. Appendix	43
Abbreviations and Terminologies	

ABSTRACT

Aging is associated with the development of vascular dysfunction, which is attributed to cardiovascular diseases (CVD) [14]. Researching possible intervention methods that promote healthy vascular aging is therefore a great biomedical priority. Essential nutrient zinc (Zn) is involved with many important biological processes including those believed to be associated with vascular function such as anti-inflammatory and antioxidant activities [1]. However, specific mechanisms are still unknown, and investigation of the effect of zinc on vascular function is limited. Using the data collected through the Integrative Physiology of Aging Laboratory (IPA), we tested the hypothesis that zinc deficiency is linked with vascular dysfunction in healthy middle age/older adults (MA/O) (n=289, Ages 50-79). Correlational analysis as well as between-group analysis (low-zinc status versus high-zinc status; low zinc-body mass ratio versus high zinc-body mass ratio) showed no significant relationship between zinc intake and direct vascular health measures such as Pulse Wave Velocity (PWV) and vasodilation measures (endothelium dependent dilation/endothelium independent dilation). However, improvements in humoral factors such as TNF- α , oxidized LDL, and NADPH oxidase were observed with higher dietary zinc intake. Although the result was inconsistent, this study supports other literature's argument on zinc's anti-inflammatory and anti-oxidant properties. Discrepancy in the result suggests the necessity for further research identifying zinc's promising capability in preventing CVD.

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I. INTRODUCTION

Cardiovascular Disease and Aging

Cardiovascular Disease (CVD) are the leading cause of morbidity and mortality in modern societies [8, 10, 14]. Out of the numerous potential physiological mechanisms thought to be causing CVD, as much as 80% are believed to be triggered by the dysfunction of arteries [13]. It has been established through many studies that aging is a major risk factor in developing CVD, as aging promotes the dysfunction of arteries through the stiffening of large elastic arteries and the development of vascular endothelial dysfunction [8, 14, 15] (Figure 1). Studying aging and the development of vascular dysfunction, as well as researching potential interventions to promote healthy aging, is clinically important.

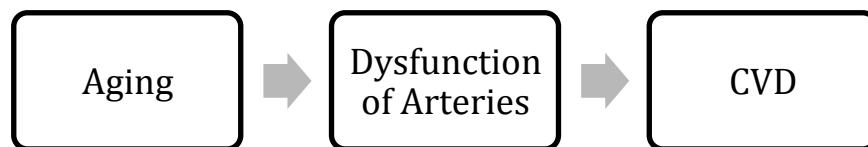


Figure 1. Basic model of aging and CVD studied at the Integrative Physiology of Aging lab. Though the complete mechanism is still unknown, it is believed that aging causes changes in biological processes that lead to the dysfunction of large elastic arteries, which consequently promotes the development of CVD.

Dysfunction of Arteries

Arteries are elastic blood vessels that carry blood pumped from the heart throughout the body in order to transport important nutrients and oxygen to the tissues. The blood exiting from the heart will first encounter large elastic arteries before it branches to successively smaller vessels. Usually, large elastic artery dysfunction refers to the stiffening of the arteries associated with the change in the structural proteins in the vessel wall [14]. When these vessels become stiffer, the pulse of blood leaving the heart travels with higher speed, which is believed to put stress on the vessels and on the end organs. Additionally, stiffening of the large elastic arteries causes the vessels to lose their ability to respond to the vasodilation (vessel expansion) signals from endothelial cells. The inability of endothelial cells to stimulate vasodilation can contribute to the development of arterial stiffness [19]. Thus, proper maintenance of the large elastic arteries becomes vital to proper physiological function. Although precise mechanism of how aging contributes to the stiffening of arteries is unknown, several evidence-supported explanations are available.

Dysfunction of Endothelial Cells

Vascular endothelial cells are a single layer of cells lining the inner surface of the arteries, and it is associated with many of the important regulatory arterial functions [14]. In fact, it is these endothelial cells that work in an autocrine and paracrine manner to regulate vasodilation [14]. Dysfunction of endothelial cells refers to the changes from normal function and phenotype of arteries that may contribute to the pathophysiological development of vascular disorders, including pro-inflammatory conditions [14]. Dysfunction of endothelial cells would therefore compromise the function of arteries [7],

and studies indicate that aging contributes to the development of endothelial cell dysfunction [1, 14]. There are several reasons why aging is believed to cause endothelial dysfunction.

Reduced Nitric Oxide Bioavailability

One of the important physiological roles of endothelium is its role in synthesizing the gaseous molecule nitric oxide (NO). NO has been studied extensively since its novel discovery in 1980 and from its recognition through the Nobel Prize in Physiology and Medicine in 1998 [7]. It is produced endogenously (synthesized by the body) by the endothelial cells, and NO stimulates the vascular smooth muscles to dilate. Studies have found that aging contributes to the reduction of NO bioavailability [7, 14], a condition being equated with “endothelial dysfunction” as it leads to decreased vasodilation capabilities [7]. Decrease in NO bioavailability with age can be attributed to the decrease in NO production, increase in NO removal, or both [14]. However, the sensitivity of smooth muscle cells to respond to NO does not change with age [14].

Oxidative Stress

Oxidative stress is the strain on the body caused by reactive oxygen species (ROS), which are molecules naturally produced by cells through cell metabolism and normal enzyme activities. Normal levels of ROS are not harmful, as ROS function to regulate cell development and are important signaling molecules [16]. However, increased levels of ROS can lead to damage and dysfunction, including the dysfunction of endothelial cells [16]. Specifically speaking, oxidative stress is believed to alter the function of endothelial NO synthase (eNOS), an enzyme that normally converts L-

arginine to NO [14]. Studies have shown that changes in eNOS function, also referred to as the “eNOS uncoupling,” can cause these altered eNOS to produce superoxide radicals instead of NO, contributing to the development of endothelial dysfunction. Furthermore, these superoxide radicals can react with NO to produce peroxynitrite, reducing the bioavailability of NO [14].

Studies show that oxidative stress is increased in endothelial cells of older adults compared to younger adult humans, which is supported in animal studies as well [1, 14]. Antioxidants, either from diet or those produced endogenously, have the ability to decrease ROS, but it has been supported that aging leads to an increased production of ROS while antioxidant protective ability is the same or decreased [14]. One of the possible mechanisms includes the action of superoxide and other free radical molecules, which are known to up-regulate NADPH oxidase, further increasing ROS activity and leading to endothelial cell dysfunction [4, 14] (Figure 2). NADPH oxidase is therefore an important source of cellular ROS in endothelial cells.

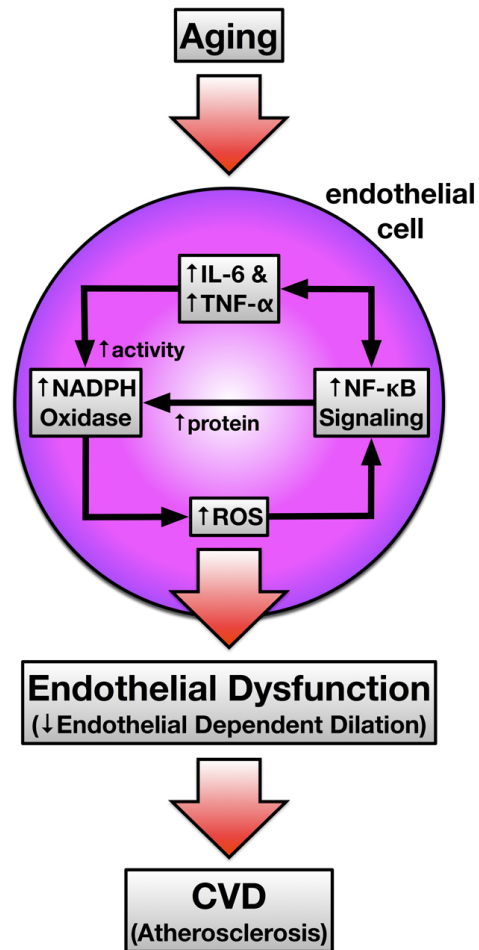
Figure 2.

Figure 2. A model illustrating the potential mechanism of how certain proteins analyzed in this experiment may be contributing to the development of endothelial dysfunction through oxidative stress [4]. A complex cross-talk between oxidative stress and inflammatory response can be observed.

“Inflamm-aging”

Chronic low-grade inflammation is associated with the constant elevation of pro-inflammatory cytokines (inflammation-inducing signaling proteins) and up-regulation of the immune system, a condition also known as “inflamm-aging.” Studies show its contribution to aging and age-related diseases, supporting that “inflamm-aging” is one of the key factors linking aging and endothelial cell dysfunction [1, 19, 20]. Inflammation is a natural physiological process for protection against harmful pathogens and healing [19]. However, just as too much oxidative stress activity can be detrimental, chronic inflammation can also be harmful. It can cause DNA damage, changes to DNA repair process, and apoptosis (regulated cell death) [19]. Specifically, oxidative stress and immune cells can activate the arterial cell wall, which can lead to the up-regulation of the cells’ pro-inflammatory gene expression (Figure 3). A study analyzing the vascular endothelial cell protein expression in older adults showed an increase in the expressions of pro-inflammatory proteins such as pro-inflammatory nuclear transcription factor-B (NFκB), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) [14]. In fact, studies show that this systemic chronic inflammation is attributed to the morbidity and mortality of elderly population [19].

Figure 3.

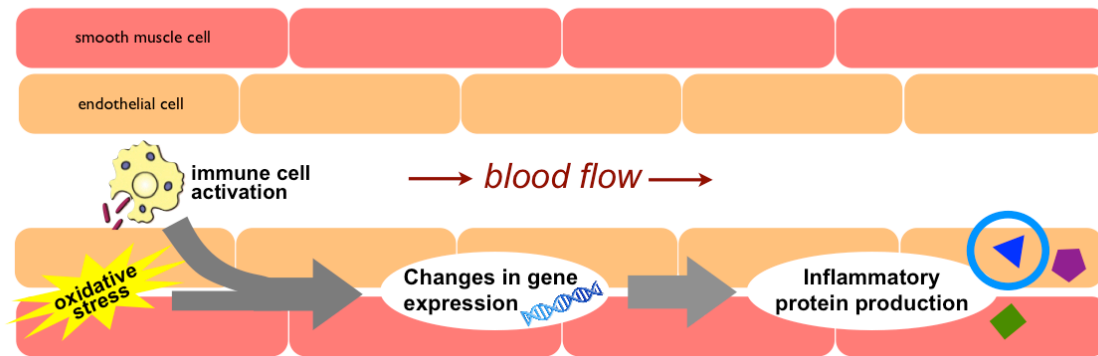


Figure 3. An illustration showing how oxidative stress and immune system activation can lead to chronic low-grade inflammation, or “inflamm-aging.” This is believed to be one of the potential causes of vascular endothelial dysfunction. This is adapted from the “You’re Only As Old As Your Arteries” presentation by Dr. Douglas Seals from the Integrative Physiology of Aging Laboratory.

Various anti-inflammatory interventions targeting “inflamm-aging” have shown that arteries, fortunately, have restorative properties. For example, inhibiting NFκB signaling has been shown to improve older adults’ brachial artery flow-mediated dilation (FMD), a measure of endothelium dependent dilation (EDD; described in **Materials and Methods**) to levels similar to young adults’ [14].

Dietary Zinc: Clinical Significance

Zinc (Zn) is an essential micronutrient mineral that functions as a catalytic, structural, and regulatory ion necessary for many important biological processes [2], including growth and development, neurological regulations, immune function, and in transcription processes [5, 19]. It is the most abundant intracellular trace element (if hemoglobin-bound iron is not considered) [2], and because the body is unable to store this mineral, the body must acquire the nutrient through diet regularly [18, 20]. Although severe zinc deficiency is a more prominent problem in developing countries, moderate low zinc status is a growing problem in developed countries as well. Approximately 12% of the population in the United States do not consume 15 milligram of zinc per day as recommended by estimated average requirement (EAR), a scientifically accepted level of nutrient intake necessary for an average individual [20]. It is of particular concern for older populations aged 50 and above, who are reported to be taking the most inadequate amount of zinc compared to other age groups [18, 19].

Biological factors as well as social factors partially contribute to this problem, as elderly populations present an inclination to consume inexpensive food lacking in micronutrients, hence lacking in zinc. Aging is attributed with reduction in appetite, teeth loss, reduction in mineral absorption, and overall reduction in caloric requirements as well, which would all contribute to the decrease in zinc intake [11]. In fact, plasma zinc levels (zinc levels of the circulation) are consistently lower in older populations [20], although evidence for changes in the absorption of zinc is inconsistent [20]. Changes in zinc physiology with aging are unclear, but zinc deficiency is a concern as it is attributed to many diseases.

Because zinc supports many of the most critical functions in human body, a small deficiency can cause problems [2]. Zinc deficiency is known to lead to conditions such as gastrointestinal disorders, renal disorders, anorexia, anemia, some cancer, AIDS, and fetal brain cell reduction [2, 5]. It is of particular interest that extensive histological, animal, and clinical research link zinc deficiency with an increase in the prevalence of aging diseases. For example, studies show that zinc-deficiency is related to age-linked pathological conditions such as immune system dysfunction, chronic inflammation, impaired adaptive immunity, and coronary disease [2, 16, 19, 20], but the specific mechanism relating aging and zinc is unclear [20]. In addition, there are studies reporting that zinc deficiency can lead to the up-regulation of pro-inflammatory cytokines and an increase in oxidative stress, which are both predicted to be involved with arterial dysfunction [1, 2]. Although extensive biomedical studies show that zinc-deficiency leads to problems relating to arterial dysfunction, the effect of zinc on vascular function remains unclear as there are no studies investigating zinc's effect on direct vascular health measures (EDD, Pulse wave velocity, etc) to my knowledge. There are only studies that investigate in-direct vascular health measures such as humoral factors (factors in the circulating system).

Zinc Intake Level and Associated Conditions

Many studies support the beneficial effect of zinc supplementation on mice and humans. For instance, one study observed an increase in the survival rate of old mice, with 50% reduction in death by infection [11]. In another mouse study, administering zinc to zinc-deficient mice showed almost 100% recovery of myocardial (middle layer of the muscle wall of the heart) functions and a two-fold decrease in arrhythmia [2]. In

humans, zinc supplementation studies have shown to improve immune function in elderly human patients with cancer, infectious diseases, and sickle cell disease, and this was associated with a decrease in oxidative stress and inflammatory cytokine levels [2, 11]. Additionally, zinc therapy has been shown to benefit the treatment of diseases such as diarrhea, leprosy, and the common cold [2].

Unfortunately, zinc supplementation studies on older adult humans commonly show inconsistency in its benefits [11]. It is recommended that adults take 15 mg/day of zinc, with an upper tolerable limit at 25 mg/day [2]. However, other studies propose that anywhere from 10 to 40 mg of zinc per day is appropriate [11]. So the recommended values are inconsistent across literature, complicating the problem.

However, literature does appear to agree that excess zinc is toxic and that it can cause zinc poisoning. Studies show that prolonged zinc supplementation or high dose supplementation even in short time period can lead to immune system inefficiency. It appears that intracellular zinc levels are kept under tight homeostasis (within 0.1-0.5 mM), so deficiency or excess can both cause problems [2]. Studies have shown that zinc toxicity can lead to an increase in apoptosis and copper deficiency [2, 11]. Toxicity-inducing zinc supplementation varies across different literature, contributing to the difficulty of researching zinc. It is relatively common to observe inconsistency in the effect of zinc across literature.

Biological Mechanisms of Zinc

As mentioned above, specific mechanisms on how zinc may be affecting biological processes relevant to aging and vascular function remains unknown. However,

various studies on zinc support two potential roles of zinc; zinc may be functioning to maintain the proper immune or antioxidant capabilities, or zinc may be functioning to maintain the proper genetic regulation in encoding proteins for effective immune system function [11].

Anti-Inflammatory and Antioxidant Roles

Many studies support that zinc may be working as an anti-inflammatory molecule, antioxidant molecule, or both [1]. Anti-inflammatory and anti-oxidant effects are separate concepts, because anti-inflammatory refers to the decrease in inflammation, whereas antioxidant property refers to the reduction of or protection against oxidative stress. However, these two actions are closely related as oxidative stress can lead to inflammation and vice versa (Figure 4). Many micronutrients enhance antioxidant enzyme function to support the immune system through regulating inflammation to appropriate levels [11], and the increasing prevalence of infection and inflammation with zinc-deficiency has supported zinc's anti-inflammatory properties [20]. There are several potential mechanisms through which zinc may have anti-inflammatory or antioxidant effects.

Anti-inflammatory properties:

In terms of the anti-inflammatory capabilities, a study showed how experimentally abolishing intracellular zinc using a zinc-chelator increased NF κ B, a pro-inflammatory transcription factor [19]. As ROS can up-regulate NF κ B, this study supports that zinc directly or indirectly decreases ROS levels or suppresses NF κ B levels. Short-term zinc supplementation to zinc-deficient mice has also shown a reduction in

pro-inflammatory response and tissue damage [19].

Antioxidant properties:

Zinc's antioxidant properties have been supported through many studies. For example, Bao observed zinc's antioxidant properties through promoting decrease in inflammatory cytokines and oxidative stress markers in older adults when 45 mg/day zinc was supplemented for six months [1]. Zinc is known to protect the cells from ROS through enhancing superoxide dismutase (SOD; reduces superoxide) and metallothionein (MT; free radical scavengers) activities, and inhibiting NADPH oxidase activity [2]. MT is a protein with high affinity to zinc that regulates zinc homeostasis, and MT-Zn complexes can protect against oxidative stress because MT releases zinc to zinc-dependent antioxidant proteins like SOD [2, 18]. An *in vivo* study showed that zinc deficiency leads to down-regulation of MT expression compared to zinc-adequate mice, supporting that zinc's antioxidant properties may be due to its contribution to MT [16]. Experiments in mouse have also shown that zinc may be contributing to the suppression of age associated oxidative stress through supporting SOD, as one type of SOD includes a zinc-dependent, cytosolic copper-zinc SOD (Cu/Zn-SOD) [15]. Therefore, without adequate bioavailability of zinc, ideal function of SOD cannot be predicted. From above evidence-supported results, it is reasonable to predict that zinc may have beneficial effects on vascular function.

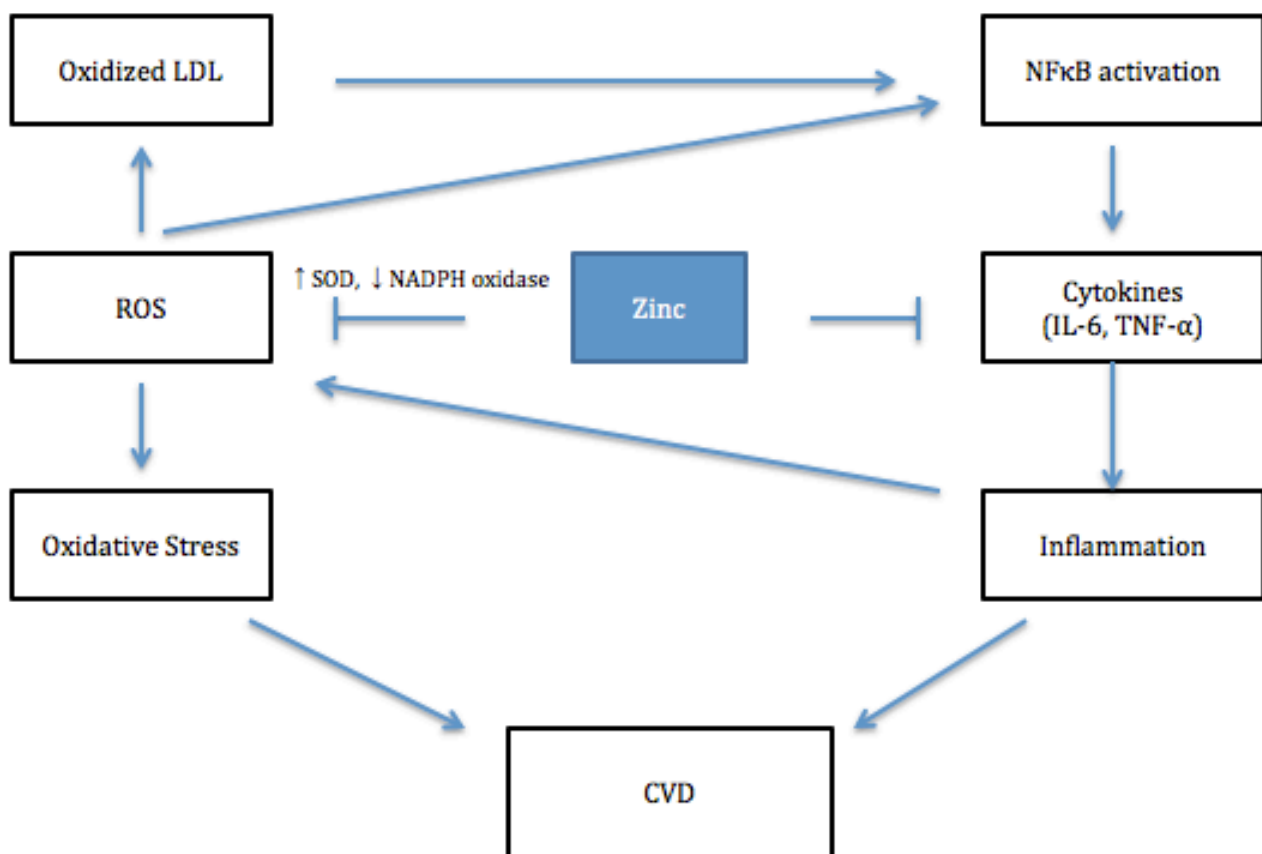
Figure 4

Figure 4. An illustration showing the potential mechanism of zinc functioning as an antioxidant and anti-inflammatory molecule in relation to factors studied in this study, as supported by studies described above. Inflammation and oxidative stress works closely together.

Transcription Level Roles

The central dogma of biology explains that DNA is transcribed to mRNA, which goes through post-transcriptional modifications to be translated to proteins. This process is one of the most important biological functions, and zinc is involved in this process as “zinc fingers” [2, 11]. Zinc fingers are projections on transcription factors (proteins that activate specific DNA sequences allowing transcription to start). Zinc fingers on the transcription factors literally bind to the DNA sequence. Without zinc, proper transcription process cannot successfully occur. Zinc is the only metal that functions as a cofactor for more than 300 enzymes [2]. Every transcription factor requires these zinc fingers, thus zinc-deficiency can cause problem in regulating this most basic and critical biological process. As an overlap from the antioxidant roles of zinc, studies have shown that long-term zinc administration up-regulates MT gene expression [2].

Working hypothesis

Although there are promising evidence for the biological benefit of sufficient zinc intake in promoting anti-inflammatory and antioxidant activities, there is surprisingly little research done on the effect of zinc on vascular health measures. Before proceeding to a zinc supplementation study, performing a preliminary retrospective analysis study using the database collected through the Integrative Physiology of Aging (IPA) Laboratory could provide insight into the relationship of dietary zinc intake and measures of vascular function. In the present experiment, we hypothesized that subjects with higher dietary zinc intake would show better vascular function measures in middle-aged and older (MA/O) individuals (Figure 5).

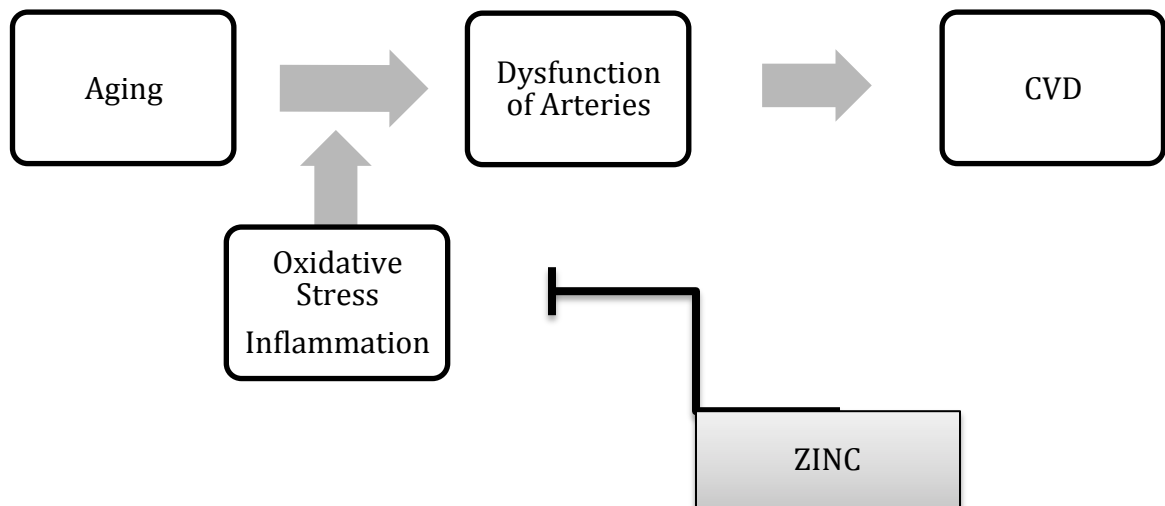
Figure 5.

Figure 5. A schematic depicting the hypothesis of this study. Although the specific mechanism is unclear, zinc may have an inhibitory effect on oxidative stress and inflammation associated with aging, which would, consequently, inhibit the development of arterial dysfunction and thus CVD.

II. MATERIALS & METHODS

Subjects

289 MA/O (50 to 79 years) men and women who were free of chronic disease, non-smokers, not exercising regularly, and were not taking any medication (over the counter or prescription) or supplements (including those with antioxidant properties) as

assessed by medical history, physical examination, blood chemistry tests, and electrocardiogram tests. The research was explained to the participants for its benefits and risks. Written consent was obtained prior to their volunteer participation in the study.

Study Procedures

All measurements used for analysis were performed at the University of Colorado at Boulder Clinical and Translational Research Center (CTRC).

Subject Characteristics and Group Selection

The dietitians from the CTRC calculated dietary zinc intake in milligrams per day based on the subjects' self-reported three-day diet record or food frequency questionnaire taken over a yearlong period. Subjects were classified into the following groups:

Categorization 1: Low-Zinc Status and High-Zinc Status

Subjects were categorized as low-zinc status (LZS) if their daily dietary zinc intake was in the bottom quartile of all subjects (daily dietary zinc intake ≤ 7.45 mg/day). Subjects were classified as high-zinc status (HZS) if their daily intake was in the top quartile (dietary zinc intake per day ≥ 15.59 mg). The cutoff values were determined using the database and not literature based zinc intake values, because:

1. Although appropriate range of daily dietary zinc is available through the literature, deficiency and excess dietary zinc, as well as zinc toxicity level intake, is inconsistent across the literature, making literature-based categorization difficult.

2. The majority of the dietary zinc intake data collected through the IPA lab are within normal and appropriate physiological level, since this experiment is not an intervention study. Using the literature based cutoff line would greatly decrease the number of subjects in each group, which would make statistical analysis difficult and make the experiment's result reliability questionable. For above reasons, quartiles were used for group categorization.

Categorization 2: Low Zinc-Body Mass Ratio and High Zinc-Body Mass Ratio

In order to control for the potential confound of differences in body mass, low and high zinc-body mass ratio groups were analyzed. The subjects were classified into low zinc-body mass ratio group (LZM) if their daily dietary zinc intake per kilogram of body mass was in the bottom quartile of all subjects (daily dietary zinc intake per kilogram of body mass ≤ 0.098 mg), and into the high zinc-body mass ratio group (HZM) if their daily dietary zinc intake per kilogram of body mass was in the top quartile of all subjects (daily dietary zinc intake per kilogram of body mass ≥ 0.222 mg).

Table 1. Subject Group Classification

	Low zinc-status (LZS)	High zinc-status (HZS)	Low zinc- body mass ratio (LZM)	High zinc-body mass ratio (HZM)
Categorization Value	Below 7.45 mg	Above 15.59 mg	Below 0.098 mg/kg	Above 0.222 mg/kg

Vascular Health Measures

Blood Assays

Blood chemistry obtained from the volunteers was analyzed for humoral factors (HDL, LDL, cholesterol, triglyceride, etc). Although these humoral factors are not a direct measure of vascular function, they give important insight into the volunteers' health status. In addition, some factors are risk factors for CVD [9].

Although arteries may not be directly available for studying, endothelial cells are available for collection and analysis, which provides a valuable opportunity to study endothelial cell physiology. For example, pro-inflammatory proteins (protein molecules that enhance inflammation including IL-6 and NFκB) levels can be analyzed. These endothelial cells are collected by inserting wires used for clinical heart catheterization procedures into the volunteers' brachial vein. Endothelial cells collected in this manner are analyzed for protein expression using immunofluorescence (fluorescent staining of specific proteins of interest).

Endothelium Dependent Dilation Measure

EDD refers to the study of peripheral vascular function through observing endothelial cell-mediated vasodilation. In other words, the response would be abolished if the endothelium were to be removed [14]. Specifically speaking, measurement methods would stimulate endothelial cells to release vasodilators such as NO, prostaglandins, and endothelial hyperpolarizing factors [15]. The primary EDD method analyzed for this study is the flow-mediated dilation (FMD).

Brachial artery FMD is a non-invasive method to measure the vascular function using a mechanical stimulus [7, 9]. The FMD works as follows: a blood pressure cuff is put on the volunteer's forearm, and the baseline brachial artery diameter is measured using an ultrasonography machine. Then, the cuff around the forearm is inflated, putting enough pressure on the vasculature to arrest the circulation to the forearm and hand. After five minutes, the cuff is deflated, which stimulates a large increase in blood flow through the brachial artery, which is a stimulus for the artery to dilate. The change in the brachial artery diameter in response to this increase in blood flow is measured using ultrasonography. The change in the vessel diameter in millimeters, as well as in percent from baseline is analyzed. Five minutes of stopping brachial artery circulation shows no evidence of harm to the volunteers [7].

In terms of the specific biological mechanism, the acute increase in blood flow induces hyperemia (excess blood in the vessels), which puts shear stress to the artery. The luminal mechanoreceptors (receptors sensing mechanical stress) send this information to the endothelial cells, which cause activation of Phosphokinase A (PKA) enzyme. PKA then phosphorylates, or activates endothelial NO synthase (eNOS) enzyme that converts L-arginine to NO. NO diffuses to the vascular smooth muscle to induce muscle relaxation, and therefore induce vasodilation [7]. Hence, the dilation depends on the activity of endothelial cell. If the endothelial cells are not functioning properly, then dilation cannot happen as effectively as the dilation of the vessel with healthy endothelium. Studies show that FMD is impaired in older adults [14], starting around the age of 40 in men and approximately 50 for women [14]. Therefore, high

magnitude in the change of vessel diameter from baseline indicates better endothelial or arterial function.

Endothelium Independent Dilation Measure:

Endothelium Independent Dilation (EID) refers to the studying of the arterial function by observing the vasodilation that occurs independent from the endothelial regulation. EID methods therefore help determine the responsiveness of vascular smooth muscle cells to signals that are not produced by the endothelium [9]. A primary method used to study EID is through the sublingual administration of nitroglycerin [14], using ultrasonography. Nitroglycerin is a “NO donor.” In other words, it provides NO directly to the smooth muscles, therefore vasodilation occurs without endothelial synthesis of NO [9]. 0.4 mg of nitroglycerin is given sublingually to the volunteers, and the change to artery diameter is measured. Improvement in nitroglycerin induced dilation can be interpreted as improvements in vascular smooth muscle responsiveness to NO.

Additionally, EID via nitroglycerin is an important measure to analyze EDD in order to make sure that the volunteer’s vessel responsiveness is proper, and to account for any group or condition differences [9]. This is possible because studies show that aging does not influence vasculature response to NO [14]. The absence of difference between groups or conditions in response to EID stimuli paired with the significant difference between the same groups or conditions in response to EDD stimuli can hence be interpreted as the presence of vascular endothelial dysfunction in vasodilation function [9, 14].

Pulse Wave Velocity

Pulse wave velocity (PWV) is a clinically important method to measure the stiffness of the large elastic arteries [15]. It measures the time it takes for the blood to travel from the carotid artery to the femoral artery (CF), or from the carotid artery to the radial artery (CR). The less time it takes for the blood to travel between these points, the stiffer the artery. Less elasticity, as mentioned in the introduction, contributes to the development of CVD. Therefore, high PWV is associated with the stiffening of the arteries, whereas low PWV is associated with less stiffening of the arteries. PWV increases with advancing age and is an independent risk factor for CVD [15].

Statistical Analysis

Statistics were analyzed using SPSS (ver. 20). Pearson correlation analysis was the primary statistical method to determine the bivariate relationships between daily dietary zinc intake or daily dietary zinc-body mass ratio with different variables of interest. Group differences were analyzed using the independent sample T-test. Statistical significance was set at $P \leq 0.05$. In biomedical physiology research, correlational importance with $r \geq 0.3$ is generally believed to be relevant. However, given the complexity of the study design in being unable to control for variables as it would have been possible to do with an intervention study, some flexibility ($r \geq 0.24$) were given in this experiment for correlational importance value. All data values presented are shown as mean \pm SE.

III. RESULTS

Subject Characteristics

Table 2. Subject Characteristics

Characteristics	All	LZS	HZS	LZM	HZM
n	289	73	72	71	71
Gender (men/women)	(159/130)	(24/49)	(38/34)	(34/37)	(32/39)
Age	60±1	60±1	59±1	61±1	60±1
Daily dietary Zn intake (mg)	12.4±0.4	5.3±0.2	22.7±0.8	-	-
Daily dietary Zn-body mass ratio (mg/kg)	0.17±0.06	-	-	0.08±0.01	0.30±0.01
Body mass (kg)	75.4±0.9	71.9±1.7	75.8±1.9	79.4±1.9	70.5±1.7
Systolic BP (mmHg)	120±1	118±2	120±2	122±2	117±2
Diastolic BP (mmHg)	74±1	72±1	73±1	74±1	71±1
BMI (kg/m ²)	25.4±0.3	25.4±0.5	25.3±0.5	27.0±0.5	24.2±0.5
Total cholesterol (mg/dL)	206±2	207±4	205±4	206±4	209±4
HDL cholesterol (mg/dL)	58±1	62±2	60±2	56±2	63±2
LDL cholesterol (mg/dL)	126±2	124±4	123±3	127±4	126±3
Triglycerides (mg/dL)	108±3	101±6	103±7	114±7	97±6

Data are mean±SE.

There were no significant differences between LZS&HZS and LZM&HZM groups except for the dietary zinc intake data. BP, blood pressure; BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein.

General subject characteristics are presented in Table 2. Mean daily dietary zinc intake for all subjects was 12.4 ± 7.3 mg (range 2 mg to 46 mg). Of the 289 subjects, 73 are included in the LZS group, and 72 are included in the HZS group. LZS had significantly lower daily dietary zinc intake compared to HZS group ($P < 0.001$). 71 subjects are classified under the LZM group, and 71 subjects are classified under the HZM group. LZM group had significantly lower daily dietary zinc-body mass ratio compared to the HZM group ($P < 0.001$). Age was inversely related with daily dietary zinc intake ($P < 0.001$, $r = -0.24$) and with daily dietary zinc-body mass ratio ($P < 0.001$, $r = -0.25$). There were no differences between paired groups (LZS&HZS; LZM&H2M) in age, body mass, systolic and diastolic blood pressure, BMI, cholesterol, lipoproteins, and triglycerides.

Blood Assays

On correlational analysis, daily dietary zinc intake and daily dietary zinc-body mass ratio were not related to SOD, Cu/Zn-SOD, IL-6, TNF- α , NF κ B expression, oxidized LDL, and eNOS (All $P > 0.05$). There were no significant group differences (LZS&HZS; LZM&H2M) on SOD, Cu/Zn-SOD, IL-6, eNOS, and NF κ B expressions (All $P > 0.05$). However, the circulating level of TNF- α level was significantly higher in the LZS versus HZS group (LZS: 1.41 ± 0.11 pg/mL vs. HZS: 0.90 ± 0.13 pg/mL, $P < 0.01$; Figure 6), although not between LZM and H2M ($P > 0.05$). TNF- α is a pro-inflammatory cytokine, and its level lowered with higher zinc intake, supporting zinc's benefit to inflammatory responses.

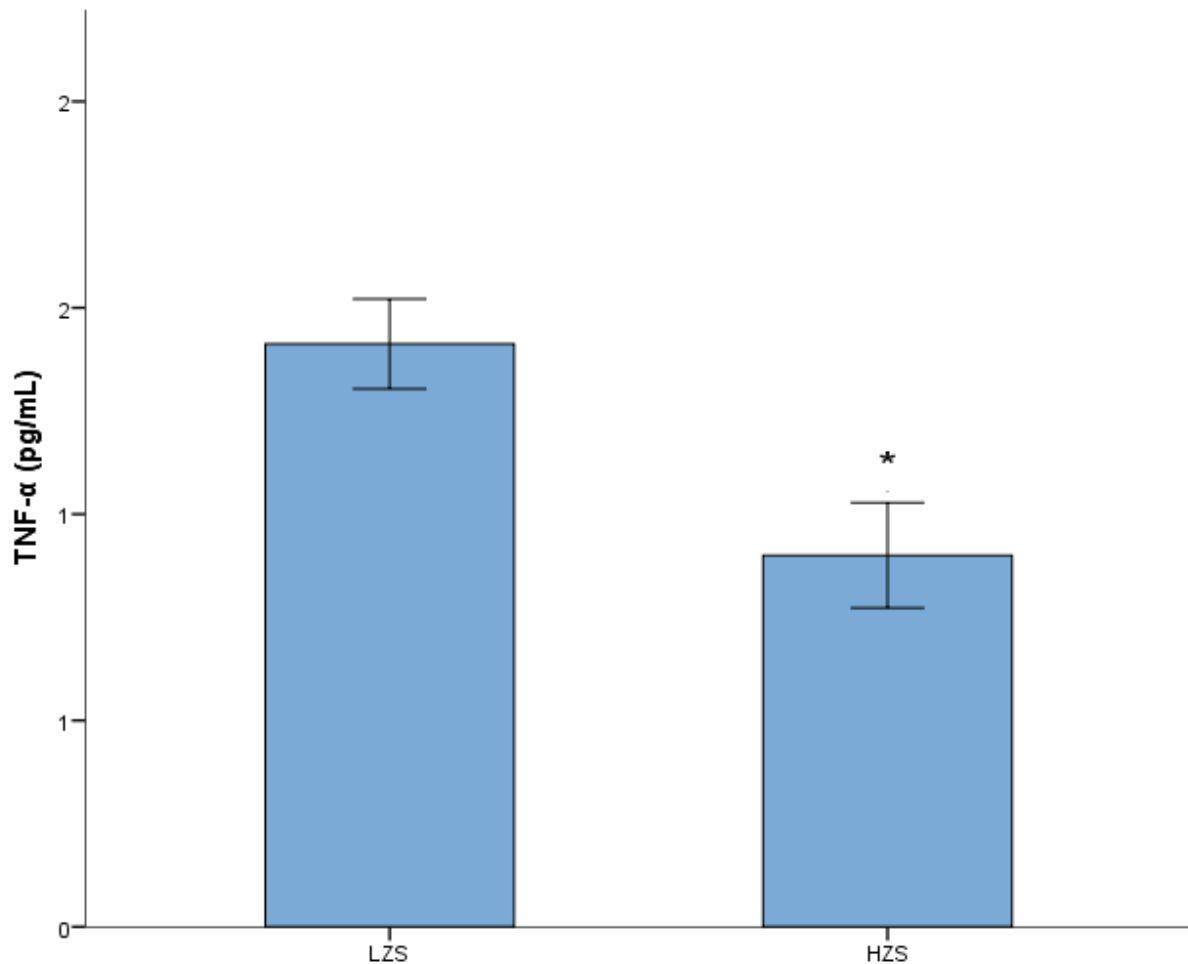
Figure 6.

Figure 6. TNF- α level between LZS and HZS was significant ($P < 0.01$). TNF- α is a cytokine involved with inflammation. Values are mean \pm SE.

In another T-test, the circulating level of oxidized LDL was significantly higher in the LZM versus HZM group (LZM: 60.58 ± 2.14 U/L versus HZM: 54.21 ± 2.13 U/L, $P < 0.01$; Figure 7), but not between LZS and HZS ($P > 0.05$). Oxidized LDL is a marker of oxidative modification of lipids, in other words, a marker of oxidative stress. Oxidative LDL level lowered with higher zinc intake, supporting zinc's benefit to reducing oxidative stress.

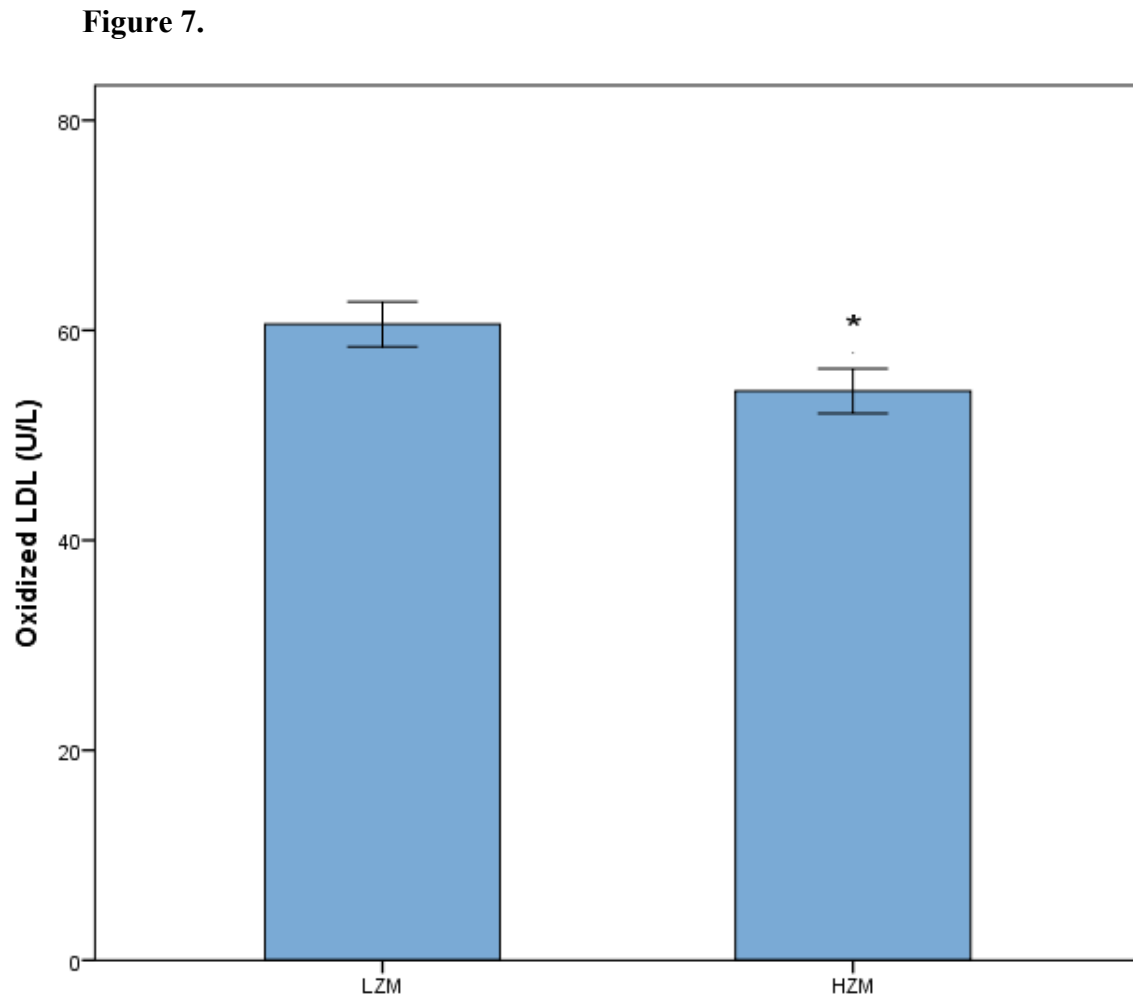


Figure 7. Oxidized low-density lipoprotein level was significant between LZM and HZM ($P = 0.03$). Values are mean \pm SE.

NADPH oxidase expression was inversely related with both daily dietary zinc intake ($P \leq 0.01$, $r = -0.34$) and daily dietary zinc-body mass ratio ($P < 0.05$, $r = -0.33$) (Figure 8).

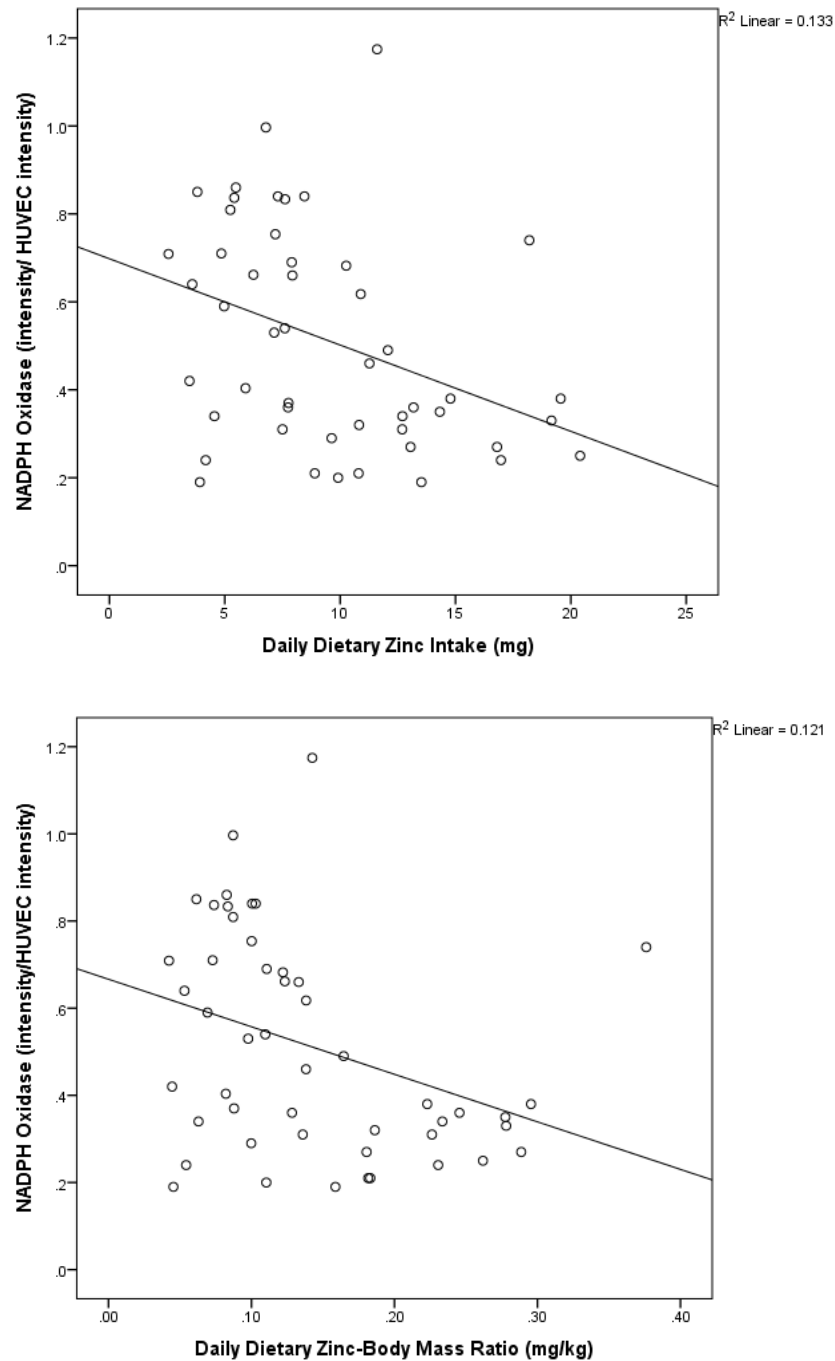
Figure 8.

Figure 8. Correlation between NADPH oxidase expression level and daily dietary zinc intake was significant for both absolute zinc intake (top) ($P \leq 0.01$, $r = -0.34$) and daily dietary zinc-body mass ratio (bottom) ($P < 0.05$, $r = -0.33$). The fluorescence is adjusted to the intensity of human umbilical vein endothelial cell (HUVEC). Both show negative relationships.

The NADPH oxidase expression was significantly lower in the LZS versus HZS (LZS: 0.72 ± 0.10 intensity/HUVEC intensity versus HZS: 0.37 ± 0.08 intensity/HUVEC intensity, $P \leq 0.05$). Similarly, NADPH oxidase was lower in the LZM versus HZM group (LZM: 0.80 ± 0.15 intensity/HUVEC intensity versus HZM: 0.36 ± 0.04 intensity/HUVEC intensity, $P \leq 0.05$; Figure 9). As described in the introduction, an increased level of the NADPH oxidase is believed to be associated with oxidative stress. The data showed a decrease in the NADPH oxidase expression levels with increasing zinc intake, supporting zinc's ability to protect against oxidative stress.

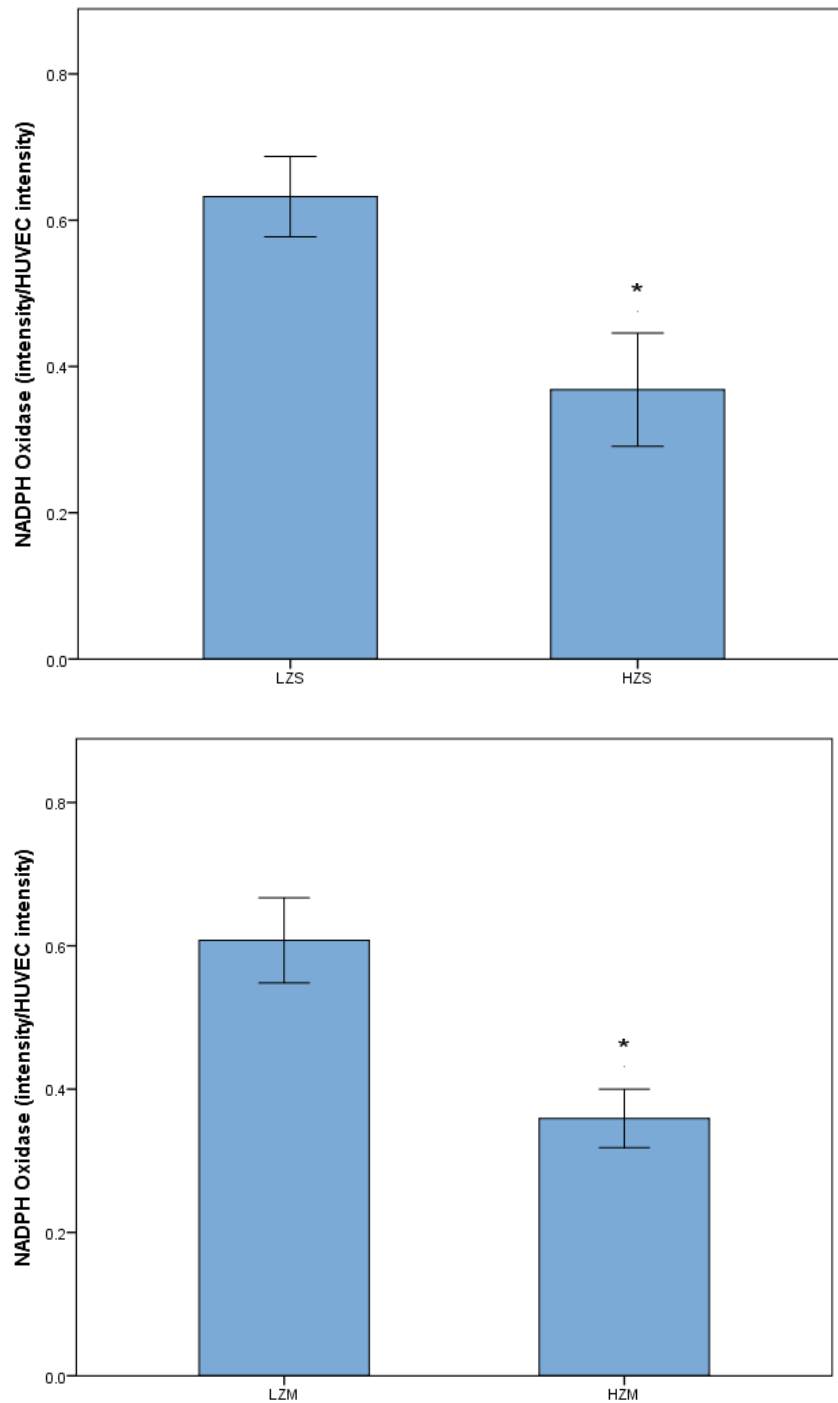
Figure 9.

Figure 9. NADPH oxidase expression level difference was significant between LZS and HZS ($P \leq 0.01$) (top), as well as between LZM and HZM ($P \leq 0.05$) (bottom). In both cases, higher zinc intake reflects lower NADPH oxidase levels. Values are mean \pm SE.

EDD and EID

EDD and EID data analysis attempt to measure the endothelial cell dysfunction by measuring the vessel dilation. Brachial artery FMD did not have a significant relationship with daily dietary zinc intake ($P > 0.05$ [% Δ], $P > 0.05$ [mm Δ]), or with daily dietary zinc-body mass ratio ($P > 0.05$ [% Δ], $P > 0.05$ [mm Δ]). FMD showed no significant difference between LZS versus HZS ($P > 0.05$ [% Δ], $P > 0.05$ [mm Δ]), or between LZM and HZM ($P > 0.05$ [% Δ], $P > 0.05$ [mm Δ]).

In EID, there were no significant relationships between the changes in arterial diameter after sublingual nitroglycerin administration with daily dietary zinc intake ($P > 0.05$ [% Δ], $P > 0.05$ [mm Δ]) or daily dietary zinc-body mass ratio ($P > 0.05$ [% Δ], $P > 0.05$ [mm Δ]). T-test difference between LSZ and HSZ was not significant, nor between LSM and HSM ($P > 0.05$ [% Δ], $P > 0.05$ [mm Δ] for both cases).

Pulse Wave Velocity

PWV measured using carotid artery-to-femoral artery (CF), and carotid artery-to-radial artery (CR) were not significantly related to daily dietary zinc intake ([CF] $P = 0.80$, [CR] $P = 0.61$; [cm/sec]), or to daily dietary zinc-body mass ratio ([CF] $P = 0.24$, [CR] $P = 0.25$; [cm/sec]). However, it should be noted that CR measure does not change with aging, as it is a measure of peripheral rather than central arterial stiffness. There were not enough data to perform a T-test for group differences between LZS&HZS and LZM&HSM.

IV. DISCUSSION

Biomedical Significance

Various histological, animal, and clinical studies suggest that zinc may have a positive role in inhibiting detrimental biological processes associated with aging and CVD [1, 2]. Literature mostly supports improvements in humoral factors associated with the function of endothelial cells, and there were no studies that evaluated direct vascular health measures to my knowledge. The data analysis from this present study matches the results from previous literature in terms of zinc's beneficial effect in improving levels of humoral factors associated with aging and CVD, although not all relations between zinc intake and humoral factors supported by the literature were significant. For example, previous studies have found that zinc deficiency leads to an increase in NF κ B levels, suggesting an inflammatory response [1, 19]. However, the result from this present study did not support any relevant correlation or group differences regarding NF κ B expression levels and dietary zinc intake.

It was of clinical importance to find that factors such as TNF- α , oxidized LDL, and NADPH oxidase levels decreased with increased zinc intake, however. Decrease in these pro-inflammatory and oxidative stress markers can be suggestive of zinc's anti-inflammatory and antioxidant properties, which are benefits thought to decrease the development of CVD. This study contributes to supporting the zinc's anti-inflammatory and antioxidant properties as reflected in other studies.

In terms of the endothelial function, EID results become relevant only when EDD results are relevant, which was not the case for this study because there were no

significant findings on EDD. Improvements to EID may suggest improved smooth muscle cell responsiveness to NO, but this was not the case either as EID result was not significant. PWV also showed no significant results. Therefore, the beneficial effects of zinc on endothelial cell function, vascular stiffness, or vascular smooth muscle responsiveness to NO cannot be supported by the data. In this study, zinc's physiological effect does not appear to be the result of other basic subject characteristics such as body mass, BMI, blood pressure, lipoproteins, and triglycerides as there were no significant group differences.

It is interesting to observe that improvements in humoral factors did not translate to the improvement in all vascular health measures (PWV, EDD/EID). There are several possible reasons for this inconsistency.

Inconsistency and Variability

A simple explanation could be that zinc does not have enough of an effect to contribute to the improvement of vascular function. However, literature supports the complexity of conducting clinical studies assessing zinc intake, which may explain the contradicting result.

First of all, as mentioned in the literature review, the tolerable lower and upper range of zinc intake is inconsistent [2, 11]. Therefore, it is very difficult to predict at which value intake can be called deficient, sufficient, and in excess. For the present study, subjects' self-reported zinc intake was used, thus the majority of daily zinc intake is within the physiological levels and not at a pharmacological level. Because most

subjects are within a narrow physiological range of zinc intake, perhaps not much biological function difference was observed.

To complicate the issue, studies support that zinc absorption ability change with age and that individual genetic variability may contribute to inconsistency in zinc absorption [11]. Also, literature suggests that zinc absorption levels vary in the type of food consumed [18]. Zinc from an animal protein source can be absorbed more easily because zinc is bound to a ligand that enhances the absorption process. On the other hand, even if zinc content is high in fiber-based food, such as rice, plants have chelating properties in abolishing free minerals including zinc, making efficient absorption difficult [18]. Some studies support that older population purposely avoid animal source of zinc such as red meat in the fear of cholesterol, and that instead they increase the intake of refined fiber products such as wheat. Although wheat is rich in zinc, the refining process depletes mineral content [18]. The magnitude as to which food source may be affecting the actual zinc absorption is unknown. Three-day diet record or the food frequency questionnaire were the main sources of calculating the subject's dietary zinc intake in this study, which means that the intake is calculated based on consumed foods. The source of zinc most likely varies among the subjects. The diet record versus the food frequency questionnaire is another source of variability. Some of the older studies run by the IPA lab used food frequency questionnaire, calculating estimate daily nutrition intake over a yearlong period. However, the diet record used for more recent studies estimates daily nutritional intake based on three days. These two methods are greatly different. Possible variability explained above may have contributed to the inconsistency shown in the data analysis.

Limitation of the Study

There are some weaknesses to the study design. Although there are many mechanisms suggested to contribute to the development of vascular dysfunction with aging, this present study concentrated on the endothelial cell dysfunction and vascular stiffness, limiting the experiment's focus. In addition, it is important to note that although this study did observe improvements in proteins associated with oxidative stress and inflammation, activity of these proteins do not directly reflect vascular function.

Additionally, according to the literature, zinc intake may not accurately represent the amount of zinc absorbed by the body for use [18]. This present study did not measure the plasma zinc level or the intracellular zinc level because such a specific measure is not performed for general studies run by the IPA lab. Although such detailed measurement would be more appropriate for intervention studies, it is still a limitation to be unable to analyze zinc levels at different degrees. Furthermore, because zinc intake is calculated through self-reported food intake, it increases the uncontrollable nature of the study.

Finally, because of the retrospective nature of this experiment, higher or lower levels of zinc intake could not be studied. A study note that dietary zinc intake within 10-40 mg/day would be appropriate [11]. However, the quartile cutoff line of daily zinc intake to categorize subjects into LZS was $Zn \leq 7.45$ mg, whereas the quartile cutoff for HZS was $Zn \geq 15.59$ mg. Given that the recommended daily zinc intake is 15 mg [2], this study analyzed group difference within a narrow range. More significant effects of zinc may be observable if a wider range of zinc intake is used for future studies.

Strength of the Study

The primary strength of this study is the large number of subjects available for analysis through the access of the IPA lab database. As presented in **Materials and Methods**, 289 subjects were available for correlational analysis. T-test group comparison studies using the bottom and top quartile for each category allowed for approximately 70 subjects as well.

V. CONCLUSION

Overall, the result of the study demonstrates that the physiological level variance of dietary zinc intake does not show significant difference in vascular health measures (PWV, EDD/EID) for healthy MA/O population. Even so, physiological level dietary zinc intake showed a significant link to improvements in humoral factors (TNF- α , NADPH oxidase, oxidized LDL). Thus, the data partially rejects and supports the hypothesis that increasing dietary zinc would improve vascular function. Zinc's effect on humoral factors are extensively supported by other literature, therefore, this experiment reflects the potential link of dietary zinc's contribution to defense against age-related vascular dysfunction and CVD.

PERSPECTIVES

This experiment provides evidence-based observation on the effect of dietary zinc intake on aging and vascular physiology. The result may not completely support the beneficial effect of zinc on vascular health. However, this does not necessarily

demonstrate that health benefits of zinc are insignificant, nor that further investigation is unnecessary. Zinc, due to its nature, frequently causes contradictory results in the clinical experiments, and perhaps a higher dose of zinc or a larger difference in zinc intake between groups may be necessary to observe significant relations between zinc intake and vascular function. Further research on the effect of zinc on vasculature with aging is therefore necessary to clarify the potential benefits of dietary zinc intake.

Investigating the effect of dietary zinc intake and vascular function in younger subjects may be a possible direction to take for future research, in order to compare the same factors analyzed in this experiment with MA/O to further examine the effect of aging.

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VII. Appendix

Abbreviations and Terminologies

Ach	Acetylcholine
CF	Carotid artery to femoral artery
CR	Carotid artery to radial artery
CTRC	Clinical and Translational Research Center
Cu/Zn-SOD	Copper-zinc superoxide dismutase
CVD	Cardiovascular diseases
EAR	Estimated Average Requirement
EDD	Endothelium Dependent Dilation
EID	Endothelium Independent Dilation
eNOS	Endothelial Nitric Oxide Synthase
FMD	Flow-mediated Dilation
HUVEC	Human umbilical vein endothelial cell
HZM	High zinc-body mass ratio group
HZS	High zinc status group
IL-6	Interleukin
IPA	Integrative Physiology of Aging Laboratory
LZM	Low zinc-body mass ratio group
LZS	Low zinc status group
MA/O	Middle aged and older adults
MT	Metallothionein
NF κ B	Nuclear transcription factor-B
NO	Nitric Oxide
PKA	Phosphokinase A
PWV	Pulse Wave Velocity
SOD	Superoxide Dismutase
TNF- α	Tumor necrosis factor alpha
Zn	Zinc